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14. ABSTRACT Identification of genes driving prostate carcinogenesis will lead to new cancer treatment. The human chromosome 8q24.21 region has been linked with increased risk for prostatic carcinoma but the how this region contributes to prostate carcinogenesis is unknown. We cloned a candidate gene, <i>POU5F1B</i> (also called POU5F1P1), in this gene desert of 1.2Mb between <i>FAM84B</i> and the <i>c-MYC</i> oncogene. <i>POU5F1B</i> is a pseudogene of embryonic Oct4 (POU5F1). A recent study found that tumor Oct4 found in prostate cancer cells is due to the gene expression of POU5F1B, not embryonic Oct4 (POU5F1). In a dataset of 171 patients, it was found that tumor Oct4 was significantly increased in primary tumors and markedly increased in metastatic tumors, when compared to normal prostate or adjacent normal tissues. Based on the analyses and our preliminary data, we think, tumor Oct4, expressed from POU5F1B in the prostate cancer susceptibility loci 8q24, is a driver of prostate tumor formation and progression, and therefore, this driver is a novel target of intervention to eliminate prostate cancer. We propose to further determine the roles of tumor Oct4 in prostate tumor formation and metastasis. We hope we can validate whether tumor Oct4 can be targeted to inhibit prostate cancer progression and metastasis. In addition, we will map out the regions critical for Oct4 to promote prostate carcinogenesis so that we can target this region to develop therapeutics for cancer treatment in the future							
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Introduction

Background: Genome-wide association studies (GWAS) have linked human chromosome 8q24.21 region with increased risk for prostatic carcinoma but the how this region contributes to prostate carcinogenesis is unknown. In this gene desert of 1.2Mb between *FAM84B* and the *c-MYC* oncogene, *POU5F1B* (also called *POU5F1P1*) is a candidate gene with coding capacity. It is a pseudogene of embryonic Oct4 (*POU5F1*). A recent study found that tumor Oct4 found in prostate cancer cells is due to the gene expression of *POU5F1P1* (Hugo name: *POU5F1B*), not embryonic Oct4 (*POU5F1*). Our *in silico* analysis found a significant increase in Oct4 (*POU5F1B*) in primary tumors and a marked increase in metastatic tumors, when compared to normal prostate or adjacent normal tissues. Tumor Oct4 expression was higher in tumorigenic prostate cancer cells than in non-tumorigenic RWPE-1 cells. Depletion of tumor Oct4 in prostate cancer cells reduced their tumorigenic potential. We cloned tumor Oct4 and found that increased expression of tumor Oct4 in prostate cancer cells stimulated tumor cell motility. Further a significant divergence was found between tumor Oct4 and embryonic Oct4 in regulating Wnt/β-caenin signaling. It is our hypothesis that tumor Oct4, expressed from *POU5F1B* in the prostate cancer susceptibility loci 8q24, is a driver of prostate tumor formation and progression, and therefore, this driver is a novel target of intervention to eliminate prostate cancer.

Objective: The objective is to determine whether tumor Oct4 promotes tumor formation and metastasis, to determine whether tumor Oct4 can be targeted to treat prostate cancer progression, and to elucidate the mechanism involved for tumor Oct4 to promote prostate carcinogenesis.

Specific Aims: 1) Investigate whether tumor Oct4 promotes prostate tumor initiation and metastasis.
2) Determine whether tumor Oct4 can be targeted to reduce prostate tumor formation, progression, and metastasis.
3) Elucidate the mechanism involved for tumor Oct4 in promoting prostate carcinogenesis.

BODY OF REPORT

Scientific portion:

Task 1. Investigate whether tumor Oct4 promotes prostate tumor initiation and metastasis.

1.1 POU5F1B gene is frequently amplified in prostate cancer especially in metastatic, castration resistant prostate cancer.

With cancer genome data increasingly available, we first determined whether POU5F1B is clinically relevant in different cohorts.

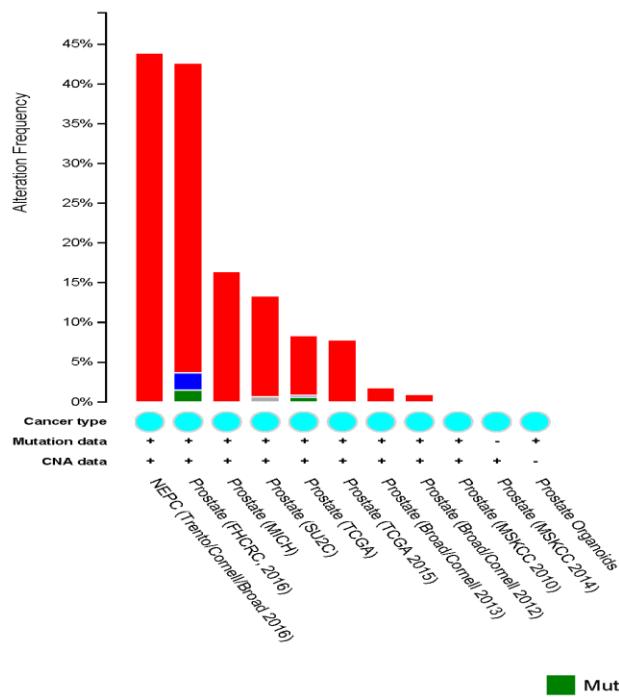


Figure 1. Amplification of POU5F1B gene loci in various cohorts of prostate cancer. Red bar, amplifications. Green bar, mutation. Blue bar, deletion. The data were obtained from cBioportal TCGA public databases.

As shown in **Figure 1**, POU5F1B gene is frequently amplified in various cohorts of prostate cancer. In the Trenton/Cornell/Broad cohort (Beltran et al., 2016), POU5F1B is amplified in about 44% of castration resistant prostate cancer specimens. Similar frequency amplification of POU5F1B was found in another cohort of metastatic prostate cancer (FHCRC) (Kumar et al., 2016). In other cohorts, the amplifications of POU5F1B are in the range between none to up to 15%, dependent upon the stages of specimens. The data suggest that POU5F1B is frequently amplified in metastatic, castration resistance prostate cancer including neuroendocrine prostate cancer.

1.2 POU5F1B expression in prostatic tissue

As cancer stem cell marker, OCT4 expression has been observed in many cancers, including breast cancer(Ezeh et al., 2005), bladder cancer(Atlasi et al., 2007), lung

cancer(Karoubi et al., 2009) and prostate cancer(Monsef et al., 2009; Sotomayor et al., 2009; Su et al., 2004). However, a recent conclusive study suggests that POU5F1B, not OCT4-A or OCT4-B, is expressed in prostate cancer cell lines and prostatic tissue (Kastler et al., 2010).

To access the clinical relevance of POU5F1B in prostate carcinogenesis, we conducted *in silico* analysis of expression profiles in a prostate cancer progression dataset (GDS2545). The dataset were obtained from 171 samples including normal, adjacent normal, primary tumors, and metastatic tumors (From left to right, **Figure 2A**). A general trend of increased POU5F1B expression was noted. Further detailed analysis of the probe intensity in the four types of tissues revealed a significant increase in POU5F1B in primary tumors and a marked increase in metastatic tumors, when compare to normal prostate or adjacent normal tissues (**Figure 2B**). It should be noted that in this dataset no POU5F1 (OCT4) expression was found. The data suggest that POU5F1B, not POU5F1 (OCT4), is responsible for prostate cancer progression.

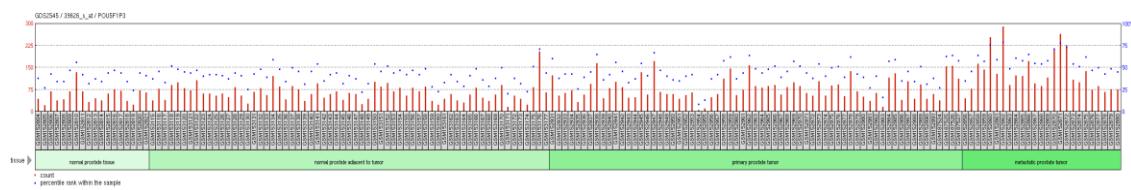
1.3. POU5F1B expression in prostate cell line and prostate carcinoma tissue

So far, six pseudogenes for OCT4 have been identified. The similarity between them and OCT4 are very high, three of them have >97%(Suo et al., 2005). The highly homologous between OCT4 and its pseudogenes can cause false positive artifacts of OCT4 expression by RT-PCR(Liedtke et al., 2008). By using OCT4 and POU5F1B specific RT-PCR, Kalster *et al.* showed it is POU5F1B, but not OCT4 is expressed in prostatic carcinoma and surrounding prostatic tissue(Kastler et al., 2010).

We can use OCT4 antibody to detect POU5F1B in prostate cell lines. First, we investigate the POU5F1B protein level in prostate tumor cell lines. Immunoblot showed POU5F1B was present in DU145, PC3, and LNCaP-T cells, a highly tumorigenic sublines of LNCaP cells. Embryonic teratoma cell, NCCIT, was used as positive control to determine the target protein band. Interestingly, POU5F1B was not detected in RWPE-1 cells, HPV-immortalized normal prostate epithelial cells (**Figure 3 A**).

Further, we examined POU5F1B expression in prostate tumor tissue by immunohistochemistry (IHC) using a validated monoclonal antibody Cell Marque MRQ-10). As shown in **Figure 3 B**, nuclear localized Oct3/4 positivity was present in the positive control (Cell Marque; CXS121). Among 44 prostate tumor tissues examined, nine had positive staining for POU5F1B. There was only one tumor section positive for POU5F1B staining out of seven prostate cancer patient specimens with the Gleason score less than three (14%), and three from 21 malignant specimens with Gleason scores from five to seven (14%). The other five tissues positive for 4 POU5F1B were from 16 prostate adenocarcinomas with Gleason scores greater than eight (31%), suggesting an increased incidence of POU5F1B positivity in high-grade prostate adenocarcinomas. In addition, the positive staining for POU5F1B in tumor specimen was mainly localized in the cell nucleus.

(A)



(B)

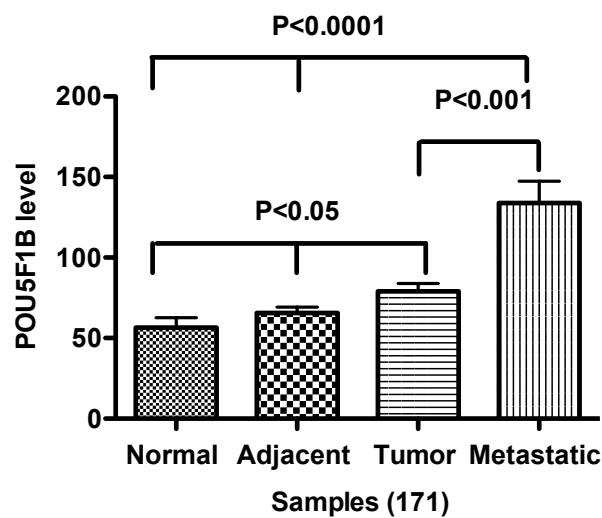
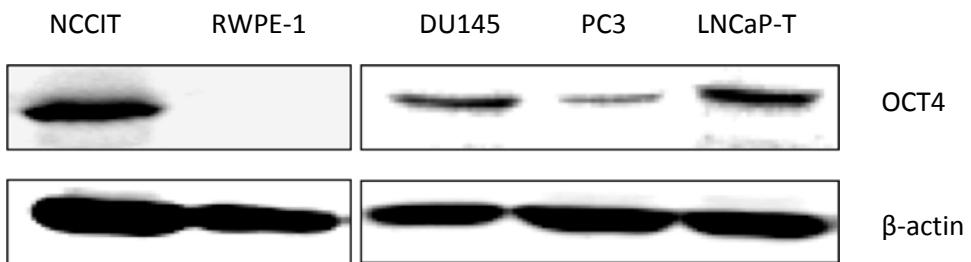


Figure 2.

tissue.

- (A) GEO profile of POU5F1B in metastatic prostate cancer (HG-U95A). Dataset type: expression profiling by array, count, 171 samples.
- (B) POU5F1B expression profiles during prostate cancer progression (GDS2545/39626_s_at/POU5F1B/Homo sapiens). Note the significant increase in POU5F1B expression in prostate tumors, especially in metastatic tumors, when compared to adjacent normal tissue or normal prostate tissue.

(A)



(B)

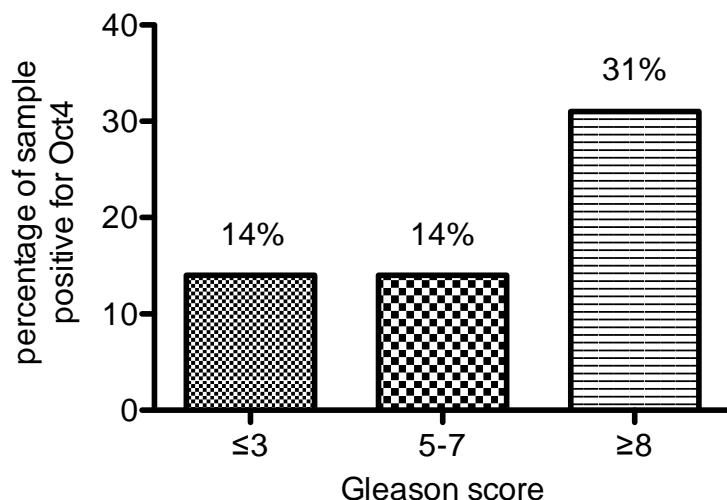
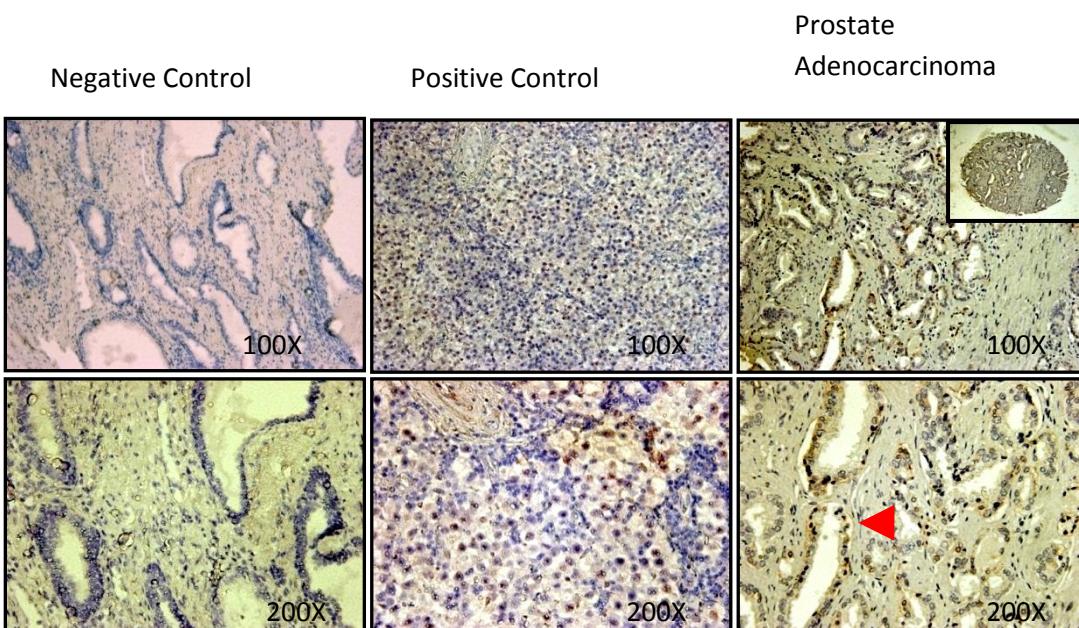


Figure 3. POU5F1B expression in prostate cell lines and prostate carcinoma tissue.

- (A) POU5F1B expression in prostate cell lines. Western blot analysis of POUF1B expression in human embryonic teratoma cell NCCIT, immortalized prostate epithelial cell RWPE-1, and prostatic carcinoma DU145, PC3, and LNCaP cells. NCCIT was used as the positive control for OCT4 expression.
- (B) Immunohistochemistry analysis of POU5F1B in prostate carcinoma samples. Left panel is the negative control. Middle panel showed positive control (Cell MARQUE, CXS121) of POU5F1B. Right panel is a malignant prostate carcinoma tissue D16 (Gleason score= 1+2) with expression of POU5F1B. Brown color indicates positive staining. Note the nuclear localization of the staining.
- (C) Statistical analysis of POU5F1B expression in 44 prostate tumor tissues. The increased incidence of POU5F1B positive staining in higher grade prostate adenocarcinomas was noted.

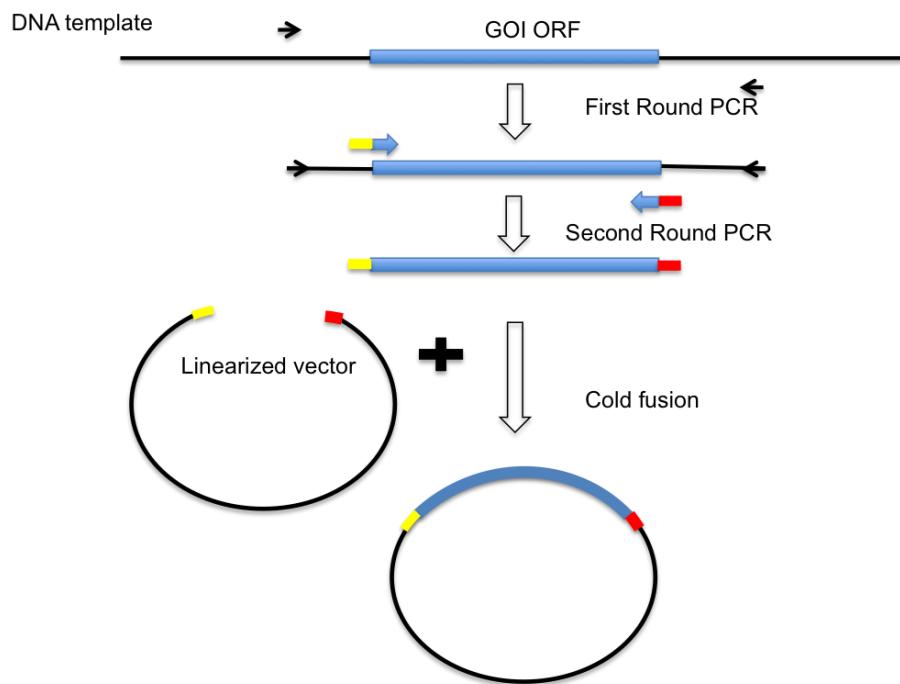
1.4. Making pCDH-myc POU5F1B construct

To further study the function of POU5F1B gene and its translated protein product, we need to generate POU5F1B overexpression construct. The expression level of POU5F1B in cells is not high, so we use Nest-PCR to amplify this gene. First step PCR enriched the fragment including our gene of interest region. Second step, we use special primer containing 20 bps homologous to linear Vector and 20 bps specific for the gene sequence. The amplified product then was cloned into the linearized vector using cold fusion kit. A schematic overview of methodical procedure is shown in Figure 3A. In our experiment, PC3 cDNA was used as template for first PCR. The products of first PCR were shown in Figure 3B left panel.

The products of second PCR were shown in Figure 3B right panel. The band was cut from the gel and extracted, and then ligated with EcoR I and Not I linearized pCDH-myc vector using cold fusion reaction.

After we get the constructs, EcoR I and Not I enzyme digestion confirmed the inserts (Figure 3B right panel).

(A)



(B)

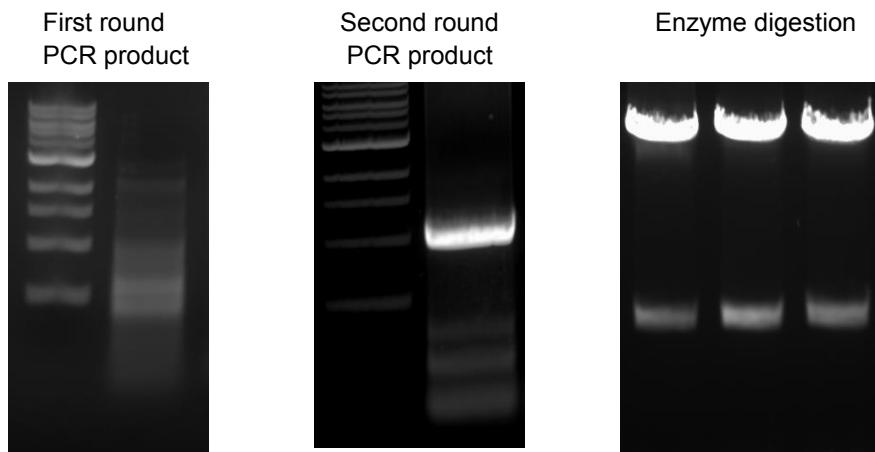


Figure 4. Cloning POU5F1B protein-coding region from PC3 cDNA into pCDH-myc Vector.

- (A) Overview of methodical procedure to make construct using nest-PCR and cold fusion cloning kit. DNA contains our target sequence are used as template, first pair of PCR primers are located 111 bps upstream of POU5F1B start codon and 122 bps downstream of POU5F1B stop codon. First round PCR product, which enriched POU5F1B sequence, was used as template for second PCR. The second pair of primers contains 20 bps homologous with pCDH-myc vector and 20 bps homologous with POU5F1B sequence. This PCR product was cut from DNA gel, and then ligated with linearized pCDH-myc vector using cold fusion kit.
- (B) PCR products of cloning steps and confirmation of the insert by enzyme digested. Left panel showed first round PCR product, which enriched POU5F1B sequence. Middle panel showed second round PCR product, which contains POU5F1B coding sequence with 20 bps upstream homologous with pCDH-myc vector and 20 bps downstream homologous with pCDH-myc vector. Right panel showed confirmation of the insert by EcoR I and Not I enzyme digestion.

1.5 Alignment of PC3 POU5F1B ORF sequence with NCBI POU5F1B sequence (NM_001159542.1)

The enzyme digestion confirmed constructs were sent for sequencing. When we aligned the sequence of insert with POU5F1B (NM_001159542.1), two mismatches was noted, CAG⁶⁸⁴→CAA⁶⁸⁴ (Glutamine/Q→Glutamine/Q) and G⁷¹²AG→C⁷¹²AG (Glutamic acid/E→Glutamine/Q). The first mismatch is a silent mutation, which will not cause amino acid change. The second mismatch will cause glutamic acid to glutamine change (Figure 4A). These two mismatches have been reported as SNP rs6998254 and SNP rs7002225 respectively (Figure 4B).

When compare PC3 POU5F1B amino acid sequence with NCBI POU5F1B amino acid sequence, only one amino acid changed, E²³⁸ in NCBI POU5F1B but Q²³⁸ in PC3 POU5F1B. When compare POU5F1B amino acid with POU5F1, we found fifteen amino acids are different, eight of them located at N domain, two located at POU specific domain, one in linker region, one in POU homeodomain and three in C domain (Figure 4C). Some amino acids change may contribute to protein structure, such as R33L, from basic, positive, polar to neutral hydrophobic; G97S, hydrophobic to polar; D108N, acid, negative to neutral; T118P, polar to hydrophobic; E135K, acidic, negative to basic, positive; T170I, polar to hydrophobic; T182K, neutral to basic, positive; Q259 polar was deleted; T351I, polar to hydrophobic (Table 1). Hans R. Schöler showed the linker between two POU domains of mouse OCT4 is exposed to the surface of the protein and it is very important for reprogramming activity of OCT4 and protein-protein interaction(Esch et al., 2013). To map the sequences or residues which are critical for the

different function between POU5F1B and OCT4 would provide more clues for better understanding why it is POU5F1B not OCT4 is expressed in prostatic carcinoma and surrounding prostatic tissue.

(A)

POU5F1B	601	CGGCCCTGCTGCAGAACAGTGGGTGGAGGAAGCTGACAACAAATGAAAATCTCAGGAGATA	660	
Insert	661	TGCAAAGCAGAAACCCTCATGCA A GCCCCAAAGAGAGAAAGCGAACCGATTC C AGAACCGA	720	
POU5F1B	661	TGCAAAGCAGAAACCCTCATGCA C AGGCCGAAAGAGAGAAAGCGAACCGATTC G AGAACCGA	720	
Insert	721	GTGAGAGGCCAACCTGGAGAATTGTTCTGCAGTGCCCACACTGCAGATCAGC	780	
POU5F1B	721	GTGAGAGGCCAACCTGGAGAATTGTTCTGCAGTGCCCACACTGCAGATCAGC	780	
Insert	781	CACATCGCCCAGCAGCTTGGCTCGAGAACGGATGTGGTCCGAGTGTGGTCTGTAACCGG	840	
POU5F1B	781	CACATCGCCCAGCAGCTTGGCTCGAGAACGGATGTGGTCCGAGTGTGGTCTGTAACCGG	840	
Insert	841	CGCCAGAAGGGCAAGCGATCAAGCAGCGACTATGCACAAACGAGAGGATTTGAGGCTGCT	900	
(B)	POU5F1B	841	CGCCAGAAGGGCAAGCGATCAAGCAGCGACTATGCACAAACGAGAGGATTTGAGGCTGCT	900
Insert	rs998254 [Homo sapiens]	TTCTCAGGGGGACCAGTGTCTTCTCCGGCCCCAGGGCCCCATTTGGT	960	
21.	POU5F1B	TAT G AAAGCAGAAACCCTCATGCA T /CGCCAGAAGGGCAAGCGATCAAGCAGCGACTATGCACAAACGAGAGGATTTGAGGCTGCT	960	
Chromosome:		8:127416550		
Gene:		LOC101930033 (GeneView) POU5F1B (GeneView)		
Functional Consequence:		intron variant,synonymous codon		
Validated:		by 1000G, by 2hit 2allele, by cluster, by frequency		
Global MAF:		G=0.4793/1044		
maghl	rs7002225 [Homo sapiens]	cpppyelcggymaycgpqvgvgvlpvpqgg		
lets	22.	llkqkrirtlgytqadvgvlglfgkv		
fsqkt	CCGAAAGAGAAAGCGAACCGATATC [C/G] AGAACCGAGTGAGAGGCAACCTGGA	vrgnlenlfqcpkptlqishiaqqlg		
lekdv	Chromosome:	talyssvpfpegevfppvsitlgspm		
hsn	8:127416578			
maghl	Gene:	maycgpqvgvgvlpqggletsqpegeagyvge		
sndga	LOC101930033 (GeneView) POU5F1B (GeneView)	rfealqlsfknmcikrlpllkwveeadnnnenlqe		
ckaetlv	Functional Consequence:	missense		
5	Validated:	by 1000G, by 2hit 2allele, by cluster, by frequency, by hapmap		
maghl	Global MAF:	C=0.4959/1080		
maghl	rs7002225 [Homo sapiens]	agspsfggqvsvflapghfgtpgygsphftals		
lets	5	5		
POU5F1	1	MAGHLASDFAFSPPPGGGDGP GGP EPGWVDPRTWLSFQGPPGGPGIGPGVGP G SEVWGI	60	
POU5F1B	1	MAGHLASDFAFSPPPGGGDGP WGA EPGWVDP L TWLSFQGPPGGPGIGPGVGP G SEVWGI	60	
PC3 POU5F1B	1	MAGHLASDFAFSPPPGGGDGP WGA EPGWVDP L TWLSFQGPPGGPGIGPGVGP G SEVWGI	60	
POU5F1	61	PPCPPPYE FC GGMAYCQPQVGVLVPQGGLETSQPE GE AGVGVESNS D GASPEPCTV TPG	120	
POU5F1B	61	PPCPPPYE L GGMAYCQPQVGVLVPQGGLETSQPE SE AGVGVESNS N GASPEPCTV PPG	120	
PC3 POU5F1B	61	PPCPPPYE L GGMAYCQPQVGVLVPQGGLETSQPE SE AGVGVESNS N GASPEPCTV PPG	120	
POU5F1	121	AVKLEKEKLEQNPE ES QDIKALQKELEQFAKLLKQKRITLGYTQADVGL TLGVLFGKVFS	180	
POU5F1B	121	AVKLEKEKLEQNPE KS QDIKALQKELEQFAKLLKQKRITLGYTQADVGL ILGVLFGKVFS	180	
PC3 POU5F1B	121	AVKLEKEKLEQNPE KS QDIKALQKELEQFAKLLKQKRITLGYTQADVGL ILGVLFGKVFS	180	
POU5F1	181	Q T T ICRFEALQLSFKNMCKLRPLLQKWVEADNNENLQEICKAETL V QARKRKRTSIENR	240	
POU5F1B	181	Q K T ICRFEALQLSFKNMCKLRPLLQKWVEADNNENLQEICKAETL M QARKRKRTSIENR	240	
PC3 POU5F1B	181	Q K T ICRFEALQLSFKNMCKLRPLLQKWVEADNNENLQEICKAETL M QARKRKRTSI QNR	240	
POU5F1	241	VRGNLENLFQCPKPTL Q IISHIAAQQLGLEKDVVVWFNCNRQKGKRSS SDY AQRED FEA	300	
POU5F1B	241	VRGNLENLFQCPKPTL * IISHIAAQQLGLEKDVVVWFNCNRQKGKRSS SDY AQRED FEA	299	
PC3 POU5F1B	241	VRGNLENLFQCPKPTL * IISHIAAQQLGLEKDVVVWFNCNRQKGKRSS SDY AQRED FEA	299	
POU5F1	301	AGSPFSGGPVSFP I APGPHFGTPGYGS P HFTALYSSVPFPEGE A FPPVS T TLGSPMH SN	360	
POU5F1B	300	AGSPFSGGPVSFP P APGPHFGTPGYGS P HFTALYSSVPFPEGE V FPPVS T TLGSPMH SN	359	
PC3 POU5F1B	300	AGSPFSGGPVSFP P APGPHFGTPGYGS P HFTALYSSVPFPEGE V FPPVS T TLGSPMH SN	359	

Figure 5. Alignment of PC3 POU5F1B with NCBI POU5F1B (NM_001159542.1) and NCBI POU5F1 (NM_002701.5).

- (A) Alignment of insert sequence cloned from PC3 cDNA with NCBI POU5F1B (NM_001159542.1) sequence. 2 mismatches were found as showed in red boxes.
- (B) Two reported SNPs in POU5F1B gene. SNP rs6998254 showed CAG⁶⁸⁴→CAA⁶⁸⁴ variant allele, and SNP rs7002225 showed G⁷¹²AG→C⁷¹²AG variant allele.
- (C) Alignment of PC3 POU5F1B putative amino acid sequence with NCBI POU5F1B putative amino acid sequence and NCBI POU5F1 amino acid sequence. Yellow highlight indicate POU specific domain, green highlight indicate POU homeodomain, and blue highlight indicate linker between these two domains. Amino acids in red mean differences between them.

Table 1 Differences in amino acid sequences between PC3 POU5F1B, POU5F1B, and POU5F1

Location	OCT4	POU5F1B/PC3 POU5F1B	Domain
22	G	W	N domain
24	P	A	N domain
33	R (Basic, Positive, Polar)	L (Neutral, Hydrophobic)	N domain
69	F	L	N domain
97	G (Hydrophobic)	S (Polar)	N domain
108	D (Acidic, Negative)	N (Neutral)	N domain
118	T (Polar)	P (Hydrophobic)	N domain
135	E (Acidic, Negative)	K (Basic., Positive)	N domain
170	T (Polar)	I (Hydrophobic)	POUs domain
182	T (Neutral)	K (Basic, Positive)	POUs domain
227	V	M	Linker
238	E(negative, acidic)	E/Q (Neutral)	POUh domain
259	Q (Polar)	–	POUh domain
314	L	P	C domain
344	A	V	C domain
351	T (Polar)	I (Hydrophobic)	C domain

We have cloned the coding region of *POU5F1B* from prostate cancer PC3 cells into a lentiviral expression vector pCDH. Sequencing revealed the presence of SNPs in the coding region of *POU5F1B* in PC3 cells when compared to the reference sequence (NM_001159542.1) but the SNPs did not alter the deduced amino acid sequence of tumor Oct4 (*POU5F1B*) (**Table 2**).

Blast with POU5F1B ORF (NM_001159542.1)			
PCDH-myc-Pou5F1B Clones	Sequenced range	Nucleotide	Amino Acid
2	1-297, 284-1080	684(CAG---CAA) 712(TCG---TCC)	no change
3	1-513 285-1080	684(CAG---CAA) 712(TCG---TCC)	no change
4	1-272 285-1080	684(CAG---CAA) 712(TCG---TCC)	no change

Table 2. Single nucleotide polymorphism in the coding region of *POU5F1B* cloned from prostate cancer PC-3 cells. Note the SNPs did not lead to a change in the amino acid sequence in tumor Oct4 (*POU5F1B*).

At the time of our study, these SNP risk associations have not been published. Complete analysis of these risk alleles with *POU5F1B* or Oct4 protein expression and transactivation activity has not been conducted yet, so the full extent of this association and functional consequences of these risk alleles is still not known. Nevertheless, we speculated that these SNPs contributed to the tumor functions of *POU5F1B* and examined the functional consequence of PC3 *POU5F1B* (which carries the SNPs present in all examined prostate cancer cell lines, rs6998254 and rs7002225) overexpression in prostate cancer cells. We cloned *POU5F1B* from cDNA of PC3 cells into a pCDH-myc lentiviral expression construct. Sequencing of our cloned insert confirmed the presence of the SNPs in PC3 *POU5F1B*, rs6998254 and rs7002225, and our PC3 *POU5F1B* protein product differs from "normal" *POU5F1B* at one amino acid residue. (**Fig.5A**) (Breyer et al., 2014).

1.6. Validation of protein coding potential of pCDH-POU5F1B construct

To determine whether if *POU5F1B* have protein-encoding potential, pCDH-myc *POU5F1B* was transfected in to 293T cells. 48 hours later after transfection, we collected lysates from the transfected cells and did western blot analysis. As shown in **Figure 6**, showed that overexpressed protein can be detected by both myc tag mouse monoclonal antibody (abm, G019, left panel) and

OCT4 rabbit monoclonal antibody (Epitomics, 2876-1, right panel). The data confirmed the protein-encoding potential of POU5F1B.

It should be noticed that POU5F1B and POU5F1 (wild type Oct4) have 95% homology in amino acid sequence (Panagopoulos et al., 2008) and therefore, many commercial antibodies against Oct4 cannot differentiate the tumor Oct4 encoded by POU5F1B from those by POU5F1. Since POU5F1 is not expressed in prostate cancer cells (in contrast to embryonic stem cells), the Oct4 detected by immunohistochemistry or Western blot is due to the expression of its pseudogene, *POU5F1B* (Kastler et al.).

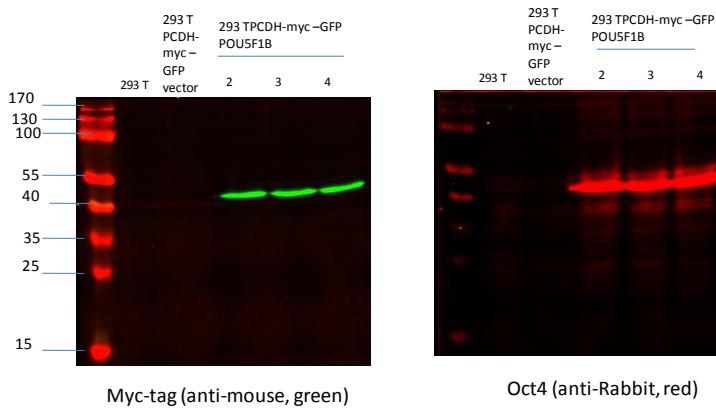


Figure 6. Validation of the lentiviral pCDH POU5F1B expression constructs. POU5F1B was expressed as Myc tag under the promoter of EF1. Left panel, Western blot with Myc tag antibody. Right panel, Western blot with an antibody against Oct4.

1.7. Overexpression of POU5F1B in prostate epithelial or tumor cells

To study the role of POU5F1B in prostate cancer progression, we packaged the lenti-virus of pCDH-myc vector and pCDH-myc POU5F1B in 293T cells, and then infected immortalized normal prostate epithelial cell, RWPE-1, and prostate cancer cell lines, LNCap, DU145, and PC3 cells. Flow cytometry was used to get the GFP positive cells. We observed the green cells under microscope (**Figure 7A**). Western blot using OCT4 rabbit monoclonal antibody (Epitomics, 2876-1) also confirmed the POU5F1B overexpression (**Figure 7B**). Immunocytochemistry of OCT4 in LNCaP and DU145 cells showed the overexpressed POU5F1B is localized in nucleus (**Figure 7**).

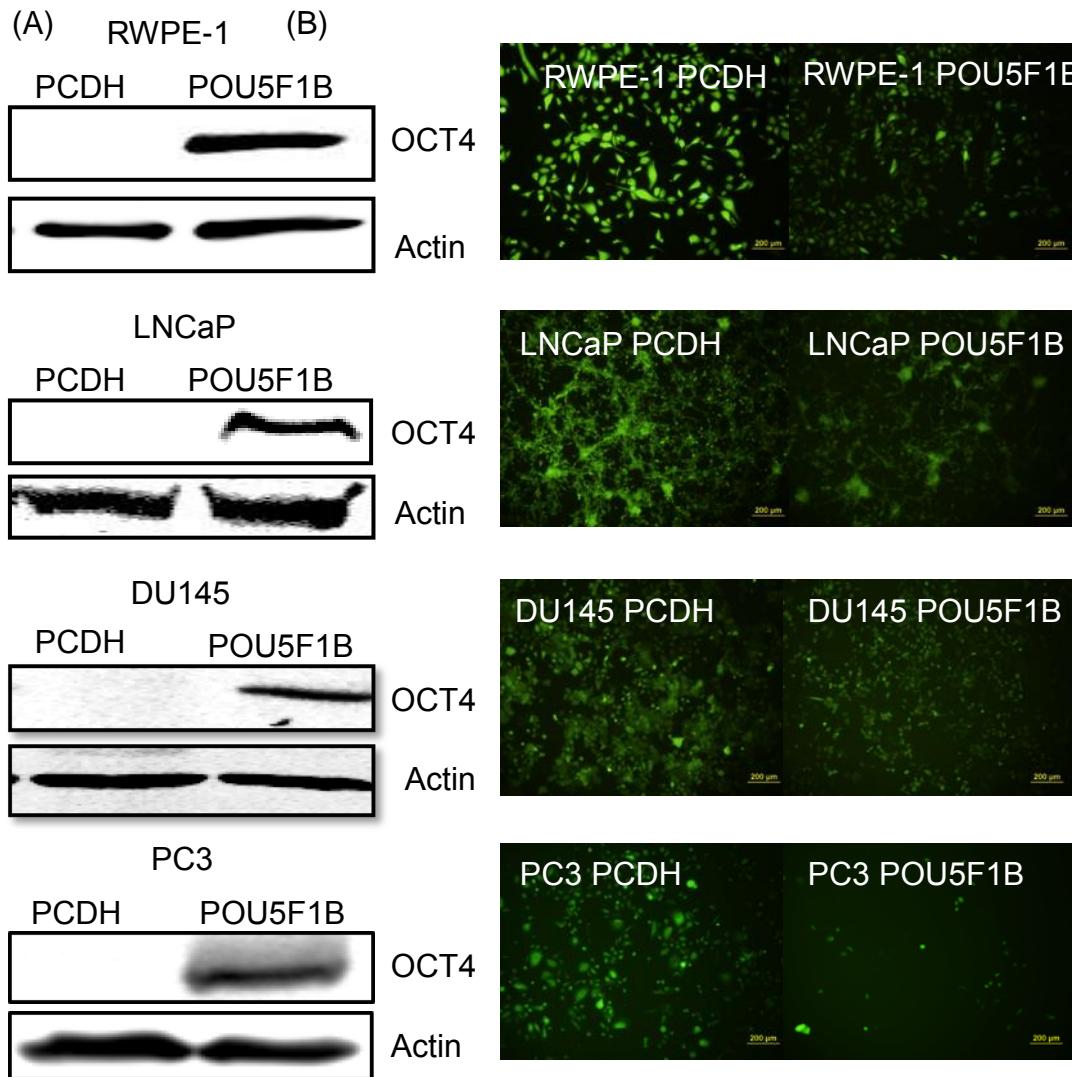


Figure 7. Establish POU5F1B overexpressed prostate cell lines.

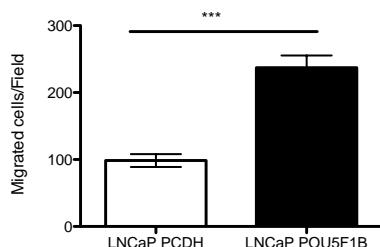
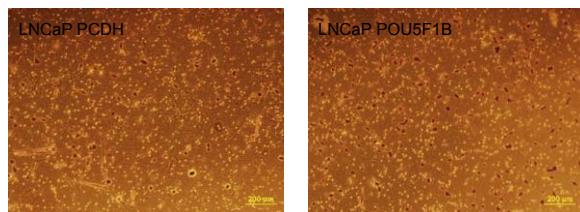
- (A) GFP was observed in cells stably infected with pCDH myc vector or pCDH myc POU5F1B under fluorescence microscope. Stably infected cells were selected by FACS.
- (B) Western blot confirmed the overexpression in RWPE-1, LNCap, DU145, and PC3 cells infected with pCDH myc POU5F1B using OCT4 antibody.

1.8. POU5F1B promotes cell migration

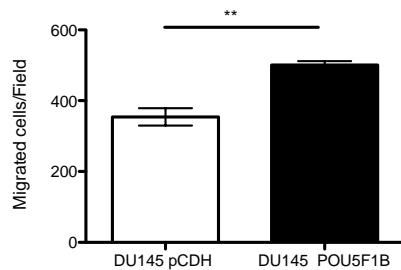
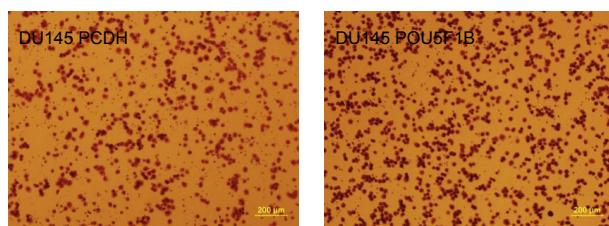
GEO data set suggests POU5F1B was robustly increased in metastatic prostate cancer tissue, so we want to test the effect of POU5F1B on cell migration. We did trans-well migration assay. In LNCaP cells, we found the number of cells that migrated through the pores in POU5F1B overexpression cells was 237 ± 18.37 , which was significantly higher than that of pCDH myc vector, 98.5 ± 9.66 ($P < 0.001$) (Figure 8A). In DU145 cells, comparing 500.5 ± 11.26 migrated

cells in POU5F1B overexpression with 354.25 ± 24.73 migrated cells in pCDH myc vector, the increasement of migrated cell number in POU5F1B overexpressed is significant ($P < 0.005$) (**Figure 8B**). We also checked the effect of POU5F1B on migration in PC3 cells. The data was consistent with the findings in LNCaP and DU145 cells. The number of cells that migrated through the pores in PC3-pCDH POU5F1B was 93.75 ± 11.16 , whereas the number of cells that migrated through the pores in PC3-pCDH myc vector was 18.50 ± 5.74 (**Figure 8C**).

(A)



(B)



(C)

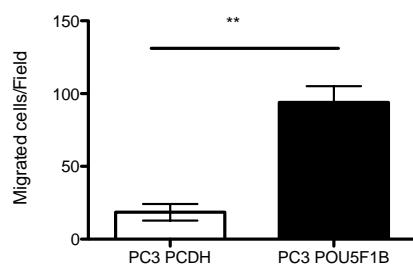
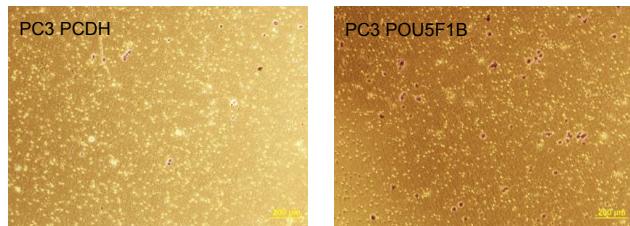


Figure 8. POU5F1B can promote cell migration.

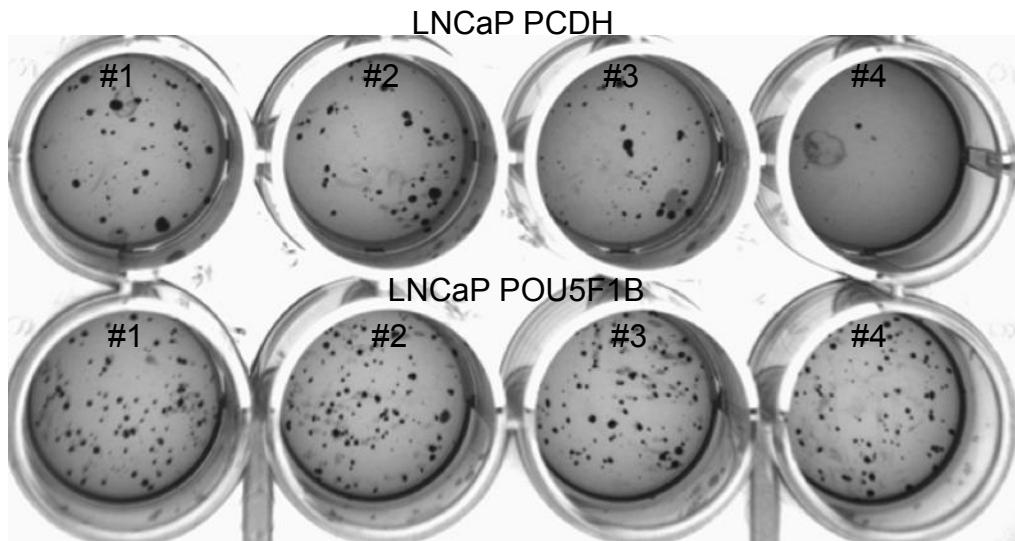
- (A) Representative images and statistically graph of cell migration in LNCaP pCDH myc vector and LNCaP POU5F1B cells.
- (B) Representative images and statistically graph of cell migration in DU145 pCDH myc vector and DU145 POU5F1B cells.
- (C) Representative image and statistically graph of cell migration in PC3 pCDH myc vector and PC3 POU5F1B cells.

1.9. POU5F1B promotes colony formation in soft agar

The anchorage-independent growth (AIG) is an important step in acquisition of malignancy(Freedman and Shin, 1974). Cells with anchorage-independent growth ability have the potential to migrate through the body, colonize other tissues and grow metastatically(Gassmann and Haier, 2008). The soft agar colony formation assay is a common method to monitor anchorage-independent growth.

As showed in **Figure 9**, LNCaP POU5F1B formed 75.5 ± 7.63 colonies in soft agar, whereas in LNCaP pCDH-myc vector, the number of colonies was significantly decreased, only 26.75 ± 7.63 ($P < 0.001$).

(A)



(B)

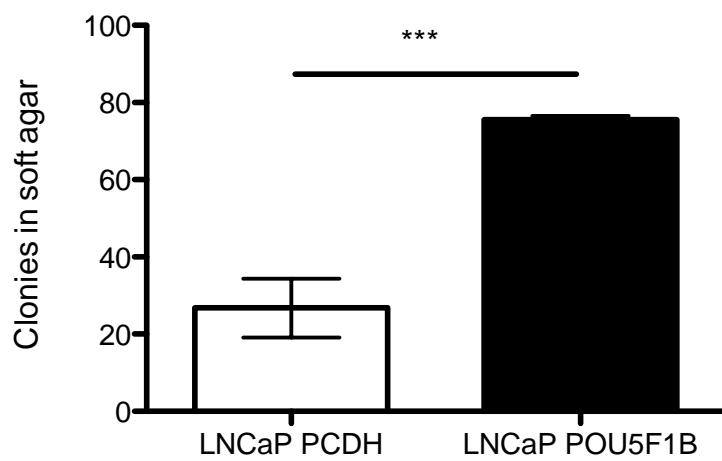


Figure 9. POU5F1B can promote colony formation in soft agar in LNCaP cells.

(A) LNCaP pCDH vector and LNCaP POU5F1B cells were seeded on soft agar and cultured for 7 days. The stained colonies were photographed, and counted.

(B) Statistical data of colonies formed in soft agar. 4 repeats for each group. $P<0.001$.

1.10 POU5F1B causes changes the morphology of DU145 cells

We noticed that DU145 cells with POU5F1B overexpression have different cell morphology when compared with DU145-pCDH myc vector cells. DU145 pCDH-myc vector showed tightly packed and formed clustered structures, typical of epithelial cells and suggestive of strong cell-cell adhesion (**Figure 10 left panel**). DU145 cells overexpressed POU5F1B showed reduced cell-cell adherens junctions and suggestive of increased cell molitity (**Figure 10 right panel**). This observation are more significant under 200 magnification. This morphology change is consistant with previous data which showed POU5F1B can promote cell migration.

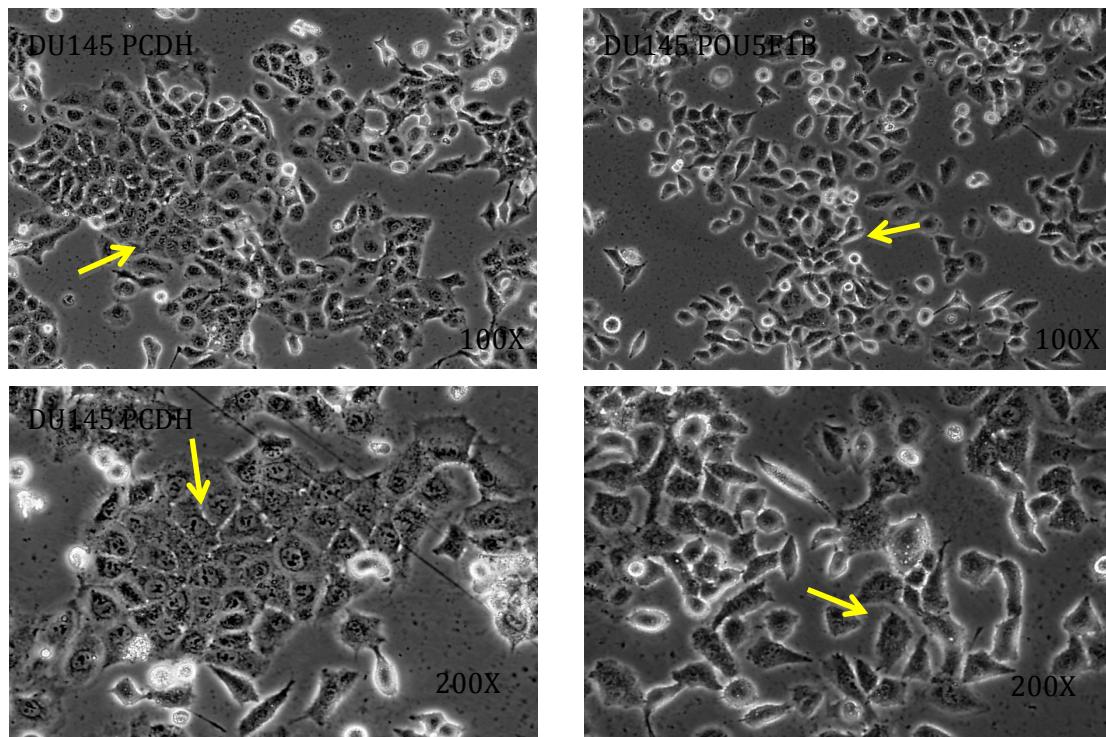


Figure 10. Comparasion of cell morphology between DU145 pCDH myc vector and DU145 POU5F1B cells. The tight cell-cell adherent junctions were found in DU145 pCDH myc vector cells, but were reduced in DU145 POU5F1B cells.

1.11. POU5F1B induces epithelial to mesenchymal transition (EMT) in DU145 cells

We observed the cells lost tight cell-cell adhesion when POU5F1B is overexpressed, which suggests the cells undergo EMT process. Down-regulation of E-Cadherin is one of the hallmarks of EMT. First, we assessed the cellular localization and expression of E-Cadherin in DU145 pCDH myc vector and DU145 POU5F1B cells. Immunocytochemistry staining showed typically E-Cadherin localization at cell-cell junction in pCDH myc vector cells, but such staining pattern was disappeared in POU5F1B overexpressed cells (**Figure 11A**). Furthermore, immunoblot confirmed the down-regulation of E-Cadherin after POU5F1B overexpression (**Figure 11B**). The reduction of E-Cadherin was not found in immortalized normal prostate epithelial cell line RWPE-1 cells with POU5F1B overexpression (**Figure 11B**). This suggest that POU5F1B decrease E-Cadherin is cell-dependent. E-Cadherin is the main component of the cell-cell adhesion junctions, loss of its expression will increase cell motility.

To investigate other EMT-related gene expression, we did EMT-related gene microarray. As showed in **Figure 12**, the epithelial marker, CDH1 gene , which encoding E-Cadherin decreased by almost 10 folds. The mesenchymal marker, CDH2, N-Cadherin encoding gene and CLDN1, claudin-1 encoding gene are increased. Twist, SIP-1 and Vimentin are the mesenchymal marker that are decreased in DU145 POU5F1B cells compared with DU145 pCDH cells.

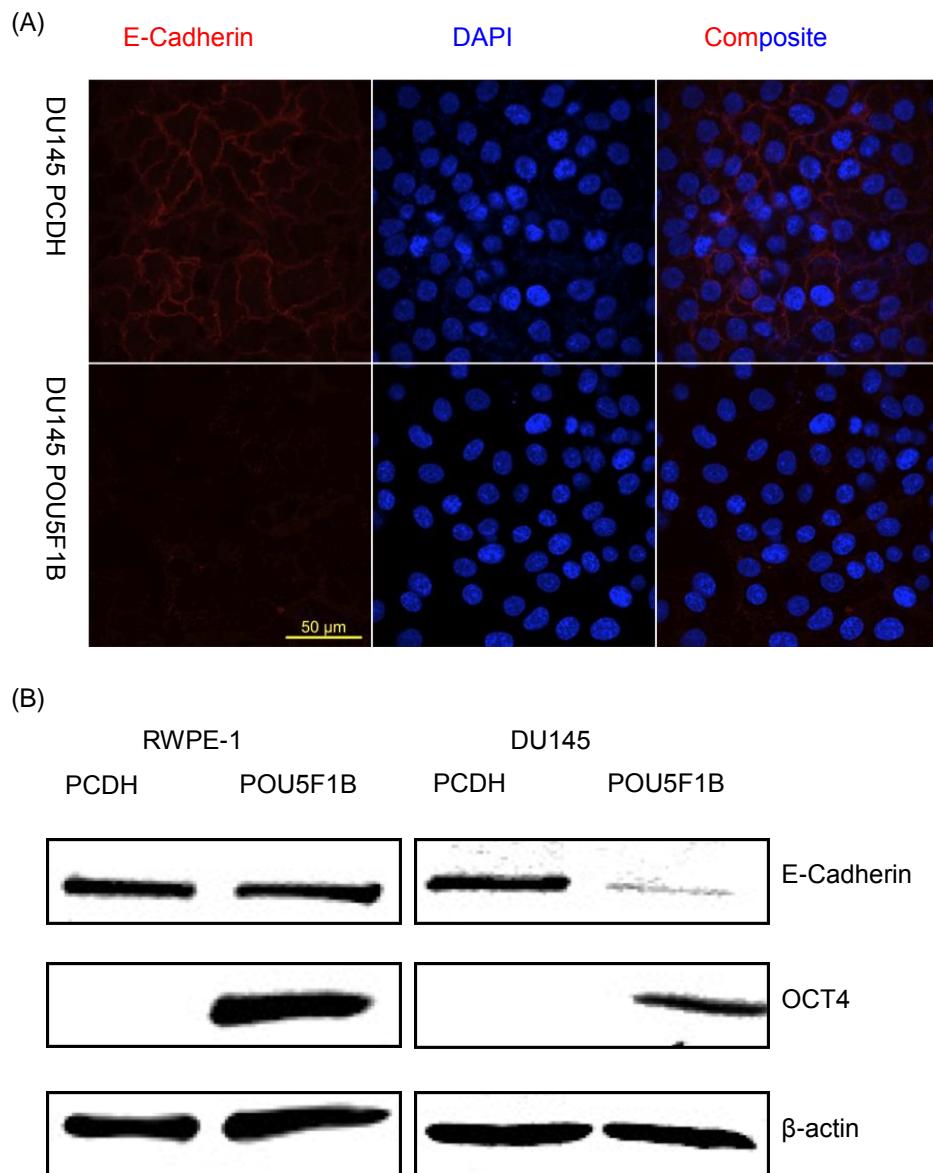


Figure 11. Effect of POU5F1B on E-Cadherin cellular localization and expression.

- (A) Immunocytochemistry of E-Cadherin in DU145 pCDH and DU145 POU5F1B cells. POU5F1B causes loss of E-Cadherin localization at cell-cell junctions. DU145 pCDH myc vector and DU145 POU5F1B cells grown on coverslips were fixed, subjected to double staining with anti-E-Cadherin (red) and DAPI (blue) and examined by fluorescence microscopy.
- (B) Immunoblot of E-Cadherin in RWPE-1 pCDH and POU5F1B, DU145 pCDH and POU5F1B cells. It was noticed that POU5F1B could decrease E-Cadherin expression in DU145 cells, but not in RWPE-1 cells.

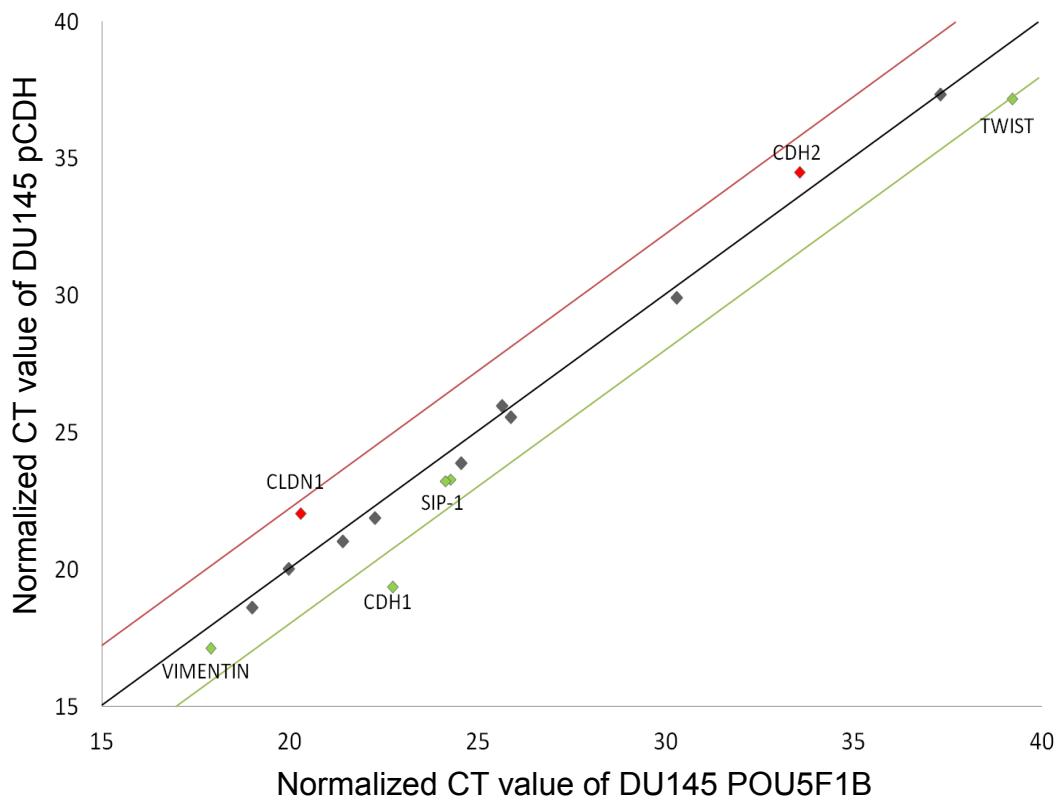


Figure 12. POU5F1B overexpression in DU145 regulates EMT-related genes expression. Scatter plot comparing EMT-related gene expression profiles between DU145 pCDH and DU145 POU5F1B cells. Red lines indicate fourfold increase in DU145 POU5F1B compared with DU145 pCDH cells. Green lines indicate fourfold decrease in DU145 POU5F1B compared with DU145 pCDH cells. CT value was normalized to beta-actin CT.

1.12. The effect of POU5F1B on tumor growth *in vivo*

We found POU5F1B, when overexpressed in LNCaP (FGC) cells, stimulated tumor growth. Due to prostate cancer heterogeneity, we wished to extend the findings to other prostate cancer cell lines. Surprisingly we found POU5F1B actually suppressed the growth of tumors from PC3 or DU145 cells.

To determine whether POU5F1B promotes tumor growth, we subcutaneously injected 2×10^6 PC3 cells with pCDH vector or POU5F1B expression, DU145 cells with pCDH vector or POU5F1B expression into nude mice. 5 mice for each group. 4 out of 5 mice formed tumor in PC3 pCDH and PC3 POU5F1B cells after injection, but tumors formed in PC3 POU5F1B cells grows much slower than PC3 pCDH cells(**Figure 13A**). In DU145 pCDH cells, 3 out of 5 mice formed tumors, and only 1 out of 5 mice formed tumor in DU145 POU5F1B cells. It is also showed that POU5F1B suppress tumor growth in DU145 cells (**Figure 13B**). POU5F1B overexpressing cells exhibit stem cell like properties. The slower tumor growth that we observed in mice is likely the result of the higher proportion of quiescent pluripotent cells, and therefore lower proportions of highly proliferative cells, that make up the POU5F1B population.

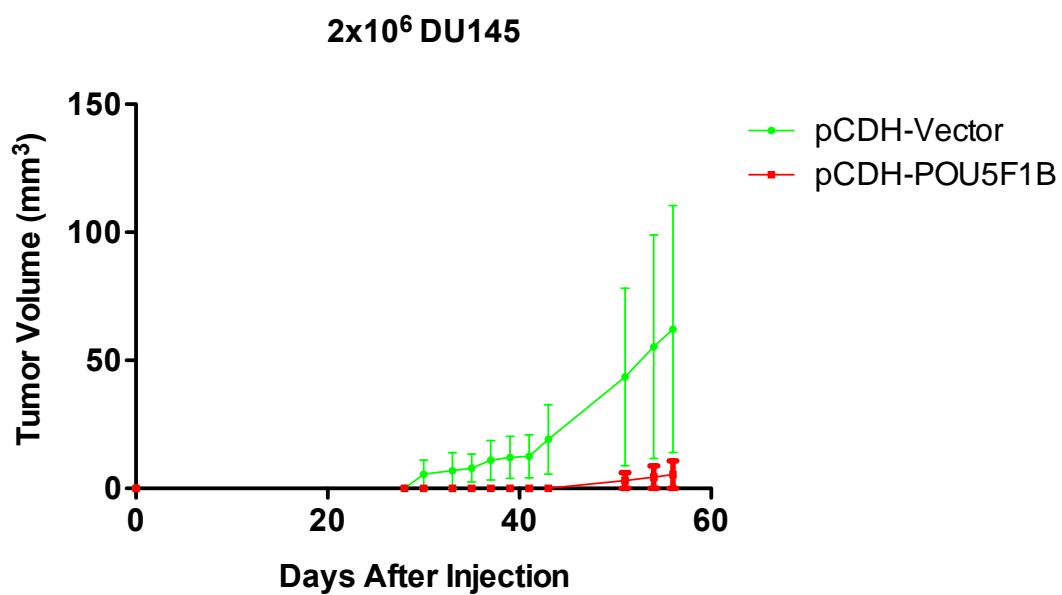
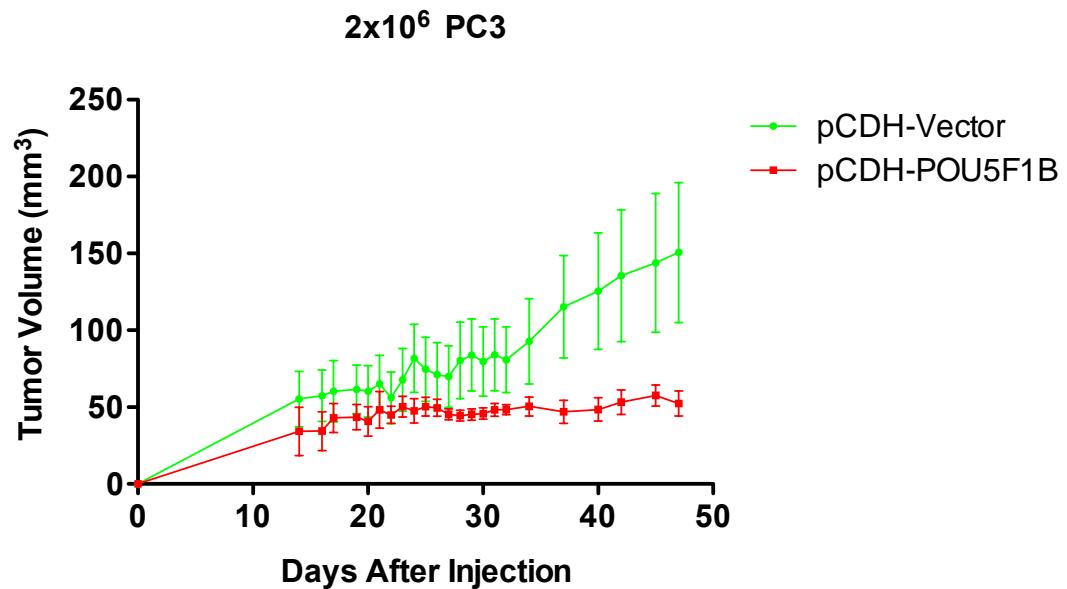


Figure 13. Suppression of tumor growth by POU5F1B in PC3 and DU145 cells.

1.13 Suppression of DU145 tumor growth, but not the formation of tumors, *in vivo* by POU5F1B

In previous reports, we found POU5F1B, when overexpressed in LNCaP-T (FGC) cells, stimulated tumor growth. Due to prostate cancer heterogeneity, we wished to extend the findings to other prostate cancer cell lines. Surprisingly we found POU5F1B actually suppressed the growth of tumors from PC3 or DU145 cells in the last report.

We repeated the studies. As shown in **Figure 14**, POU5F1B indeed suppressed the growth of DU145 tumors, but not the formation of tumors.

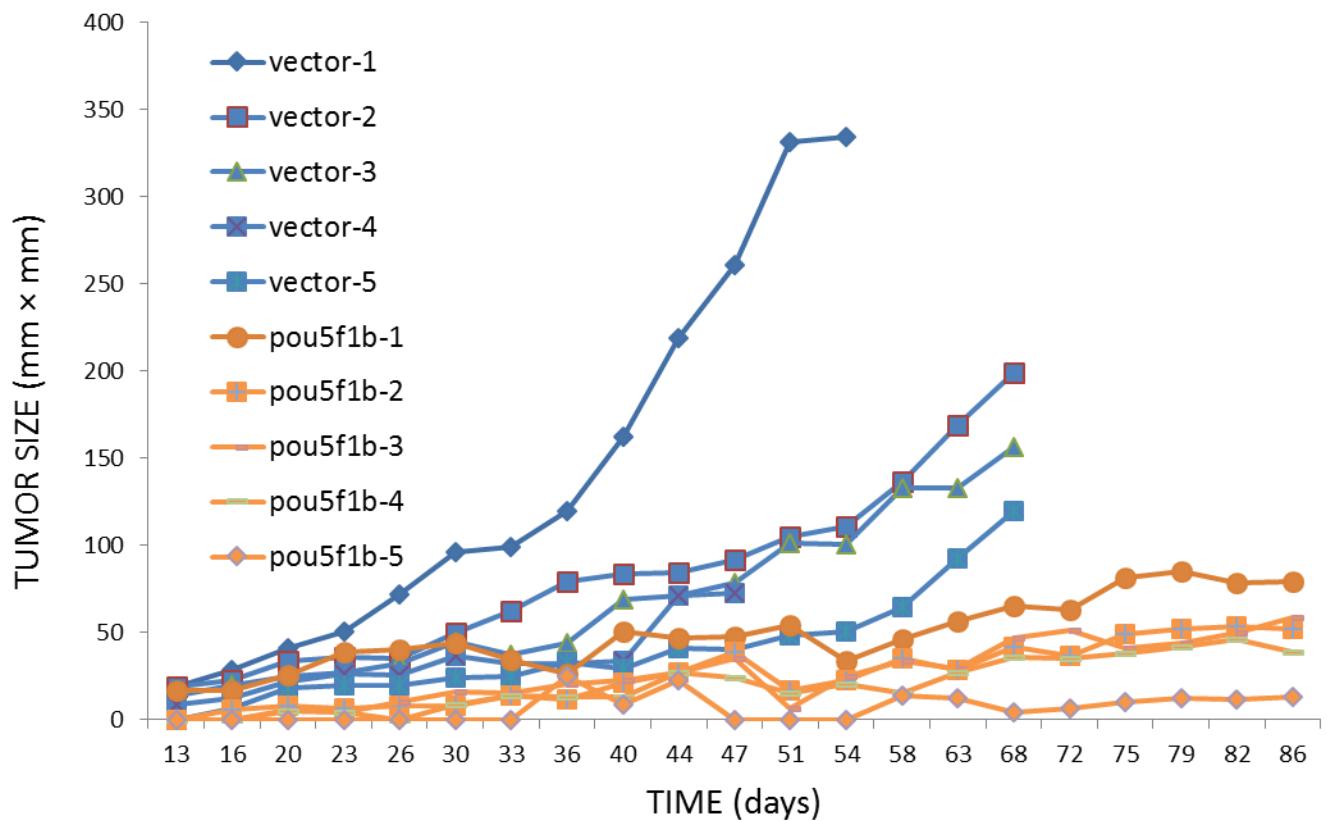


Figure 14. Suppression of DU145 tumor growth by POU5F1B.

Task 2: Determine whether tumor Oct4 can be targeted to reduce prostate tumor formation, progression, and metastasis.

We have introduced shRNA constructs into PC3MM and DU145 cells. Currently we are screening stable clones that can be used for our studies. Interestingly we found that in addition to POU5F1B, POU5F1, the putative gene for Oct4, is also expressed in the prostate cancer while we were characterizing the clones. This raises two questions: First, PCa cells may express

POU5F1, in addition to its pseudogene. Second, knockdown of POU5F1B, may have stimulated the expression of POU5F1. Since the shRNAs cannot differentiate POU5F1B from POU5F1, this complexity confound the interpretation of the data.

Currently we are using an alternative approach, the CRISPR gene editing approach, to delete POU5F1B, to determine whether its deletion can lead to reduced tumor formation, and metastasis. Additional funding are being sought to fund the alternative approach.

Task 3: Elucidate the mechanism involved for tumor Oct4 in promoting prostate carcinogenesis.

3.1. POU5F1B and MYC were co-amplified in same subset of prostate cancers

We analyzed the patterns of POU5F1B amplifications in association with clinical parameters such as cancer type, tumor sites, and ploidy. As shown in **Figure 15A**, POU5F1B amplifications can be found both in castration resistance prostate cancer (CRPC) adenocarcinoma and CRPC neuroendocrine (NE) cancer in the Trenton/Cornell/Broad cohort (Beltran et al., 2016). The amplifications seem associated with those specimens with increased ploidy, suggesting that the gain of chromosome or chromosome segments likely lead to POU5F1B amplifications. Since MYC gene, like POU5F1B, is also located in the chromosome 8q24 region, we determined the pattern of MYC amplifications in the same cohort. As shown in the figure 2A, MYC is also frequently amplified in the similar subsets of tumor specimens from castration resistance prostate cancer.

Similar co-amplifications of POU5F1B and MYC were also found in the FHCRC cohort of metastatic prostate cancer (Kumar et al., 2016) (**Figure 15B**). The data suggest that the POU5F1B is frequently amplified with MYC gene loci in more than 40% metastatic or castration resistant prostate cancer.

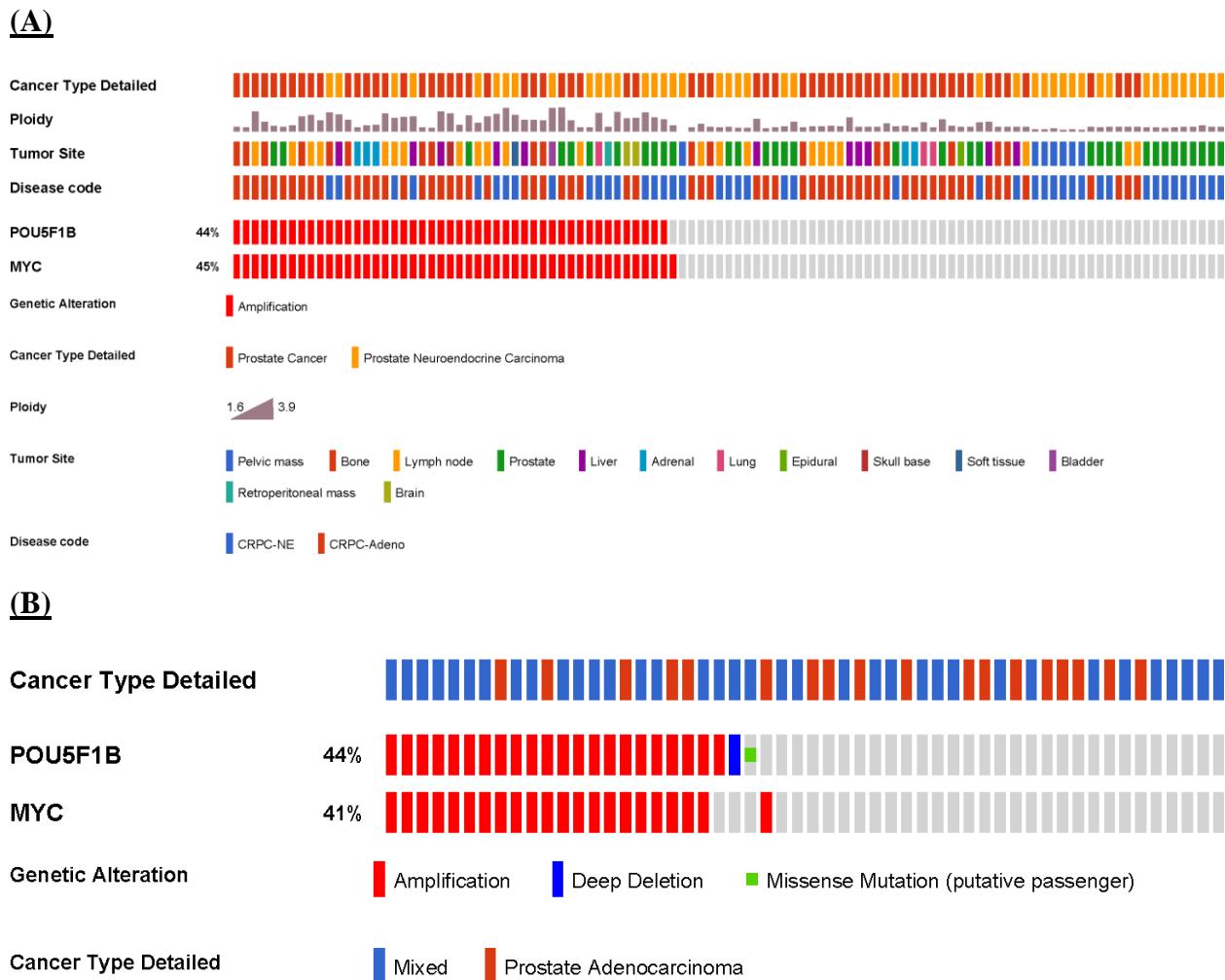
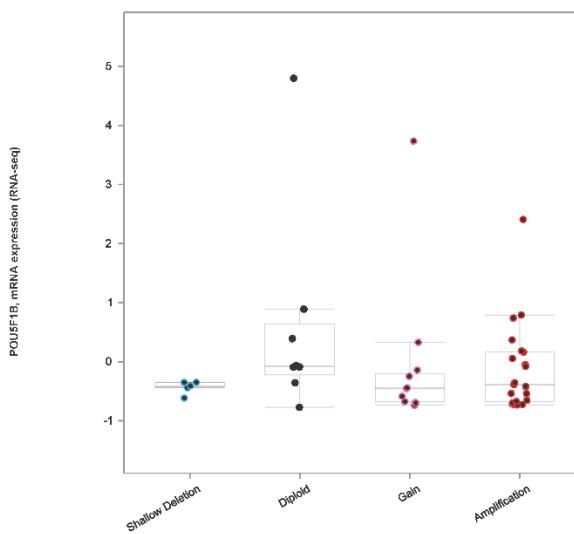


Figure 15. Co-amplifications of POU5F1B and MYC in castration resistant prostate cancer (CRPC) or metastatic prostate cancer.

3.2 POU5F1B expression in prostate carcinoma tissue is associated with androgen receptor (AR).

We detected Oct4 protein expression in prostate cancer cells as well as in tumor tissue specimens. With the availability of RNAseq data from the Trenton/Cornell/Broad cohort (Beltran et al., 2016), we first checked whether amplifications of POU5F1B can lead to increased POU5F1B expression. As shown in **Figure 16A**, amplifications of POU5F1B gene loci did not necessarily lead to its increased expression, as observed in metastatic prostate cancer. The data suggest that other factors are required for increased POU5F1B expression in metastatic prostate cancer.

(A)



POU5F1B, Putative copy-number alterations adjusted by ploidy...

(B)

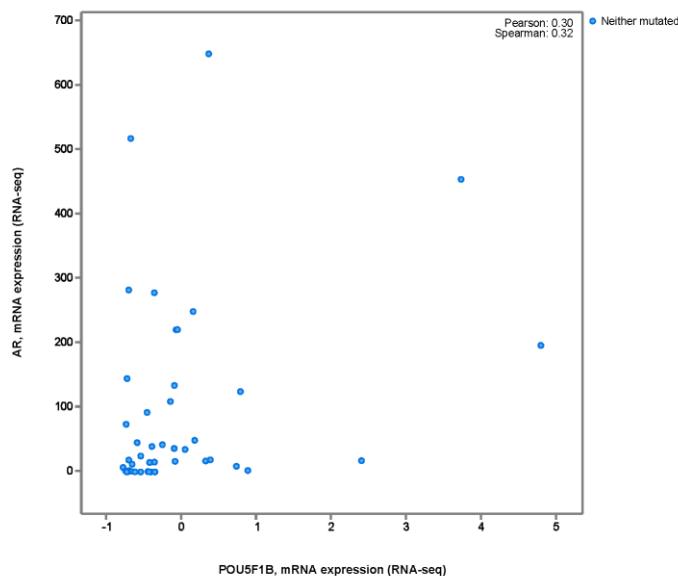


Figure 16. POU5F1B expression in prostate carcinoma tissue (A) and its association with AR (B).

Next we determined whether POU5F1B is associated with androgen receptor (AR) since it plays an important role in castration resistance and metastatic progression of prostate cancer. As shown in Figure 16B, there is correlation between these two in terms of the expression at RNA levels,

with Pearson and Spearman coefficients as 0.30 and 0.32, respectively. The functional significance of the correlation needs further studies.

3.3. POU5F1B and stemness

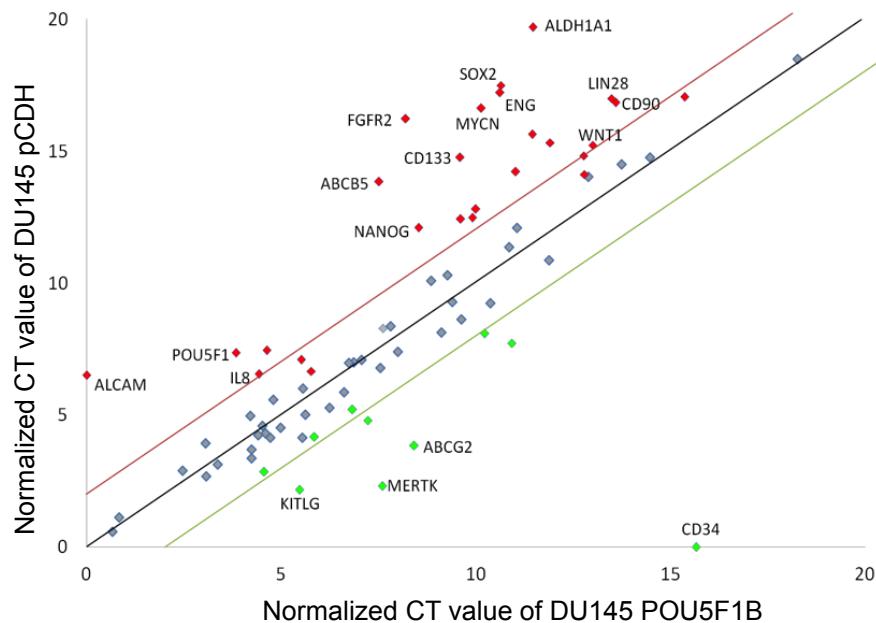
To investigate whether POU5F1B overexpression can induce cancer stem cell-related genes expression, we did cancer stem cell microarray. 84 cancer stem cell related genes are investigated. The functional grouping of these genes were shown in **Table 3**. We observed a very prominent overexpression of cancer stem cell related genes after POU5F1B expression. In DU145 cells, nearly half of all tested genes were overexpressed by at least 2 folds in POU5F1B cells. In PC3 cells, 2 fold overexpression was observed in a quarter of tested genes. Most notably, we found 14 genes that are significantly overexpressed in both DU145 and PC3 POU5F1B cells, including essential pluripotency regulators NANOG, SOX2, and BMI; cell adhesion and migration molecules PECAM1, THY1, ITGA4; and oncogenes MYCN, cKIT, WNT1 (**Figure 17**).

Table 2. Functional grouping of cancer stem cell microarray genes

Table 3

Functional group	Genes
Cancer Stem Cell Markers	ABCB5, ALCAM, ALDH1A1, ATXN1, BMI1, CD24, CD34, CD38, CD44, ENG, ETFA, FLOT2, GATA3, ITGA2, ITGA4, ITGA6, ITGB1, KIT, MS4A1, MUC1, PECAM1, PROM1, PTPRC, THY1
Proliferation	EGF, ERBB2, KITLG, LIN28B, NOS2
Self-Renewal	BMP7, DNMT1, FGFR2
Pluripotency	KLF4, LIN28A, MYC, NANOG, POU5F1, SOX2
Asymmetric Division	FOXP1, HDAC1, MYCN, SIRT1, WNT1.
Migration & Metastasis	AXL, ID1, IL8, KLF17, PLAT, PLAUR, SNAI1, TWIST1, TWIST2, ZEB1, ZEB2.
Loss of Stemness	ALDH1A1, CD34, DACH1, FOXA2, PECAM1, PTCH1.
Signal Transduction Pathways	
Hippo Signaling	LATS1, MERTK, SAV1, TAZ, WWC1, YAP1
Hedgehog Signaling	PTCH1, SMO.
Notch Signaling	DLL1, DLL4, JAG1, MAML1, NOTCH1, NOTCH2
WNT Signaling	DKK1, EPCAM, FZD7, WNT1
PI3K/AKT/mTOR signaling	ABCG2, GSK3B
STAT/NFKB Signaling	IKBKB, JAK2, NFKB1
Therapeutic Targets	ABCG2, ATM, AXL, CHEK1, DDR1, DKK1, EPCAM, FZD7, GSK3B, ID1, IKBKB, JAK2, KLF17, NFKB1, PTCH1, SMO, STAT3, TGFBR1, WEE1

(A)



(B)

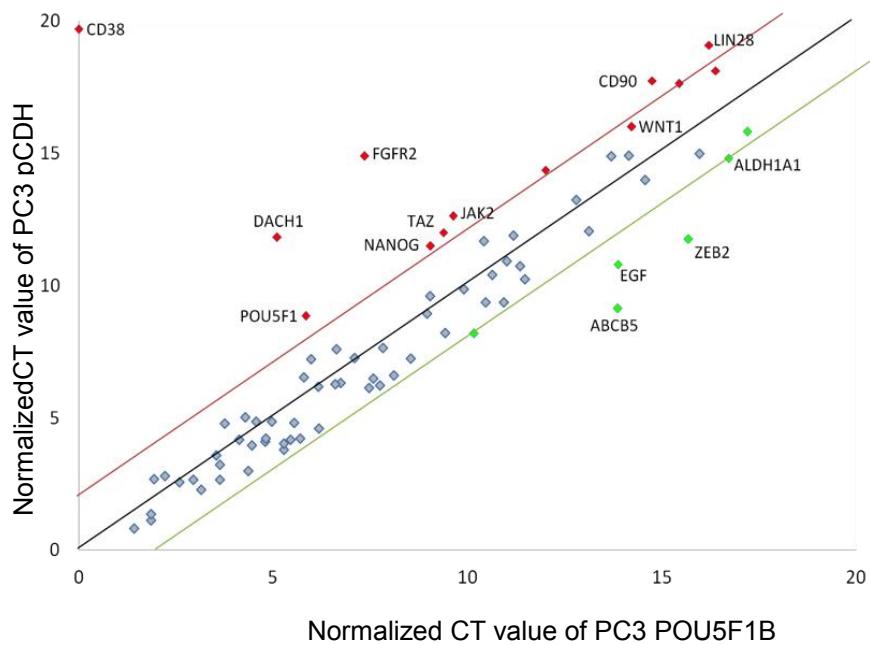


Figure 17. Scatter plot of cancer stem cell genes expression profile between cells with pCDH vector and POU5F1B expression. Red lines indicate fourfold increase in POU5F1B compared with pCDH cells. Green lines indicate fourfold decrease in POU5F1B compared with pCDH cells. CT value was normalized to house-keeping gene CT.

(A) Cancer stem cell genes expression profile in DU145 pCDH and DU145 POU5F1B.

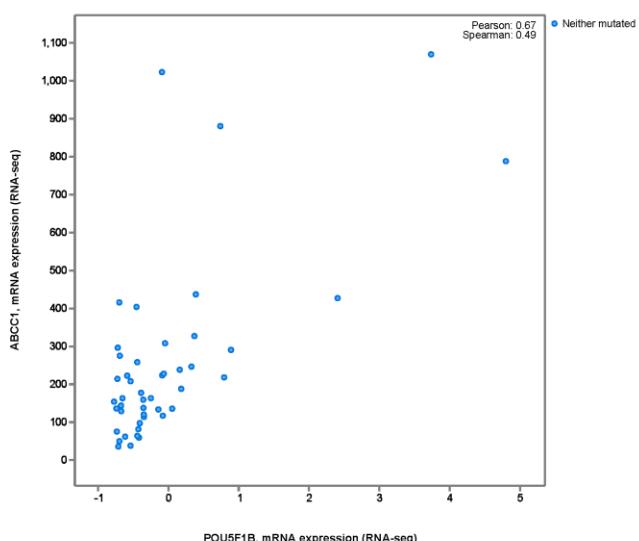
(B) Cancer stem cell genes expression profile in PC3 pCDH and PC3 POU5F1B.

3.4. POU5F1B association with ATP-binding cassette efflux transporters

The data above suggest that POU5F1B induces many stemness gene signatures in DU145 and PC-3 cells. The changes suggest that POU5F1B may have reprogrammed DU145 and PC-3 stem-like phenotypes: The ability to form tumors, but not necessarily to stimulate tumor growth.

One of characteristics for cancer stem cells is the resistance toward therapeutics. The drug resistance is partially mediated by the slow cycling cells and/or ATP-binding cassette (ABC) efflux transporters. We next studied whether POU5F1B expression is related to ABC transporters. As shown in **Figure 18**, POU5F1B is associated with ABCC1 and ABCC4. ABCC4 is found upregulated in prostate cancer.

(A)



(B)

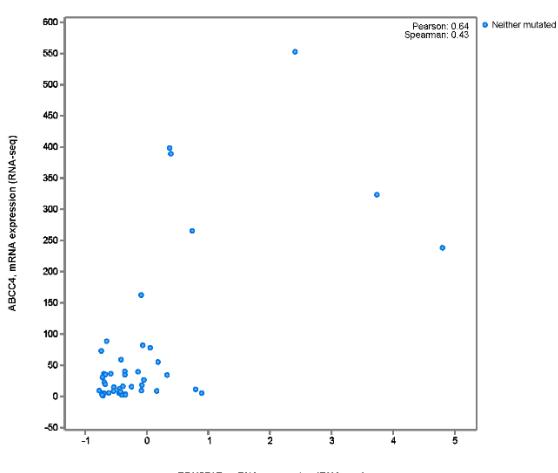


Figure 18. Association of POU5F1B with ABC efflux transporters ABCC1 (A) and ABCC4 (B).

KEY RESEARCH ACCOMPLISHMENT and REPORTABLE OUTCOMES

Presentations and abstracts:

Hongmei Jiang, Man-Tzu Wang and Daotai Nie. The Role of POU5F1B in Prostate Cancer. AACR 2013 Annual Meeting, Washington, DC, 2013.

Hongmei Jiang, Man-Tzu Wang, and Daotai Nie. The Role of POU5F1B in Prostate Cancer. Simmons Cancer Institute 2013 Research Symposium, Springfield, IL, October 2013.

Hongmei Jiang, Man-Tzu Wang, Jiuwei Wang, Yande Guo, Yuanqin Zhang, and Daotai Nie, POU5F1B, an OCT4 Retogene, Suppresses Uncontrolled Tumor Growth. Keystone Meeting on Molecular and Cellular Basis of Growth and Regeneration (A3), Breckenridge, Colorado USA, January 2016.

Abstracts published:

Hongmei Jiang, Man-Tzu Wang and Daotai Nie. The Role of POU5F1B in Prostate Cancer. Proc. Amer. Assoc. Cancer Res. 52 Late breaking: LB-281, 2013.

Review article published:

Man-Tzu Wang, Hongmei Jiang, Debasish Boral and Daotai Nie. Cancer Stem Cells in Resistance to Cytotoxic Drugs: Implications in Chemotherapy. B. Bonavida (ed.), Molecular Mechanisms of Tumor Cell Resistance to Chemotherapy, Resistance to Targeted Anti-Cancer Therapeutics 1, DOI: 10.1007/978-1-4614-7070-0_8, Springer Science+Business Media New York 2013.

Research articles published:

The manuscript requires further revisions.

Conclusions and significance (So what?):

Our studies found that:

1. POU5F1B, the pseudogene of POU5F1 localized in prostate cancer susceptibility loci 8q24, is frequently amplified in castration resistant metastatic prostate cancer.
2. POU5F1B expression is associated with AR expression level expressed in prostate cancer.
3. The expression of POU5F1B was found increased in prostate tumors when compared to adjacent normal tissues or normal prostate tissues and it was markedly increased in metastatic prostate carcinoma, compared to primary tumors or normal prostate tissues.
4. POU5F1B was cloned from prostate cancer cells. Sequencing found two SNPs when compared to the reference sequence, but the SNPs did not cause amino acid sequence changes.
5. Prostate cancer cells with increased POU5F1B expression had increased migratory phenotype and decreased E-cadherin expression at protein or mRNA level.
6. POU5F1B effect on tumor growth can be context dependent. In some cells (LNCaP), POU5F1B promoted tumor growth but in other cells (PC-3 and DU145), POU5F1B suppressed tumor growth, but not the formation of tumors. The divergence may be due to their differential dependence on AR signaling.
7. POU5F1B expression led to increased “stemness” signature and associated expression of ABC transporters ABCC1 and ABCC4.

Our studies suggest complex roles of POU5F1B in prostate carcinogenesis. More studies are needed to determine when and how POU5F1B can be targeted to reduce prostate cancer metastasis.

APPENDICES

N/A

SUPPORTING DATA

Embedded in the reporting body

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